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An *in vitro* corneal model with a laser damage threshold at 2 µm that is similar to that in the rabbit

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ABSTRACT

Corneal organotypic cultures were generated as per existing methods, which included growth on polycarbonate inserts and air-lifting for one week. The corneal simulant cultures were exposed, with real-time IR imaging, to the 2-µm wavelength output of a thulium fiber laser with 4 mm beam diameter for 0.25 seconds in a thermally controlled environment and then assayed for damage. The *in vitro* threshold (ED_{50} value of 12.5 W/cm^2) and peak temperature (77.5 °C) at threshold irradiance are compared with rabbit corneal data in the literature.

Keywords: corneal organotypic culture, laser, threshold, thermography, Probit

1. INTRODUCTION

Use of lasers has become commonplace, due to the many applications for intense, focused light sources. These uses include medicine, military, and scientific applications, to name a few. For this reason, it is important that safety standards be properly defined to allow for maximum protection, while still allowing for safe use of lasers in the desired application. In common practice, the maximum permissible exposure (MPE) limit is defined in the American National Standard for Safe Use of Lasers (ANSI Z136.1-2007)[1]. MPEs are typically around 10x the estimated dose irradiance required to achieve damage with 50% probability (ED_{50}), as determined by a threshold study conducted using an approved *in vivo* model. The purpose of this paper is to introduce an *in vitro* model that closely mimics the behavior of a similar *in vivo* model, specifically for threshold determination.

An *in vivo* threshold study conducted using dutch-belted rabbit cornea as the model system is compared with thresholds obtained using corneal organotypic cultures generated using methods described by Robertson *et al.*[2]. The irradiance threshold (ED_{50}) and peak temperatures at threshold and at upper and lower fiducial limits are given and discussed, with respect to the animal study. We believe that the *in vitro* model is in good agreement with the *in vivo* model. In addition, the use of an *in vitro* model bypasses some of the difficulties created when using an animal model. This opens the door toward conducting more detailed and prolonged experiments, which would otherwise not be feasible when using an *in vivo* model. Future use of this *in vitro* model could aide in the determination of damage mechanisms and support computer modeling efforts, due to the increased simplicity and ease with which this model system can be obtained and maintained.

2. METHODS

2.1 Corneal Organotypic Simulants

Human telomerase-immortalized corneal epithelial (hTCEpi) cells (kind gift of Dr. J.W. Shay[2]) were cultivated as suggested.[2] Stock cultures were grown in 75-cm^2 flasks and subcultured (split ratio of 1:10) every 3 - 4 days. Cultures were maintained at standard conditions (37 ^oC and atmosphere containing 5% CO_2) in KGM-2 medium (Clonectics, BioWhittaker, Inc., Walkersville, MD). To generate organotypic constructs, hTCEpi cells (giving 5 X 10^4 cells/cm^2) suspended in 0.2 ml KGM-2 culture medium containing 1.15 mM calcium were seeded into cell culture inserts (6.5-mm diameter, 3.0 µm pore size, Becton Dickinson and Company, Franklin Lakes, NJ). The inserts were then placed into 24-well plates having 0.6 ml of KGM-2 culture medium containing 1.15 mM calcium. The medium in the culture insert (0.2

[i]Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the United States Air Force.

ml) and culture well (0.6 ml) were changed every other day for 7 days. To induce differentiation into corneal simulants, the medium was removed from the culture insert (air-lifting), exposing the cells to an air-liquid interface. The medium in the cell culture well was changed every day for the next 7 days during air-lifting. After the 14 days in culture, the air-lifted corneal simulants were ready for laser exposure experiments.

2.2 Optics Layout and Laser Exposures

Corneal simulants were exposed to the CW output beam of a rack-mountable thulium fiber laser (IPG Photonics Corporation; Oxford, MA; Model: TLR-20-2000-LP) at a wavelength of 2 µm. Figure 1 shows the optics layout for the experiment. Prior to experiments each day, a ratio of laser power was generated by measurement at both the position of the cells and at a reference detector (after splitting a small portion of power with a thin piece of BK7 glass) located prior to a mechanical shutter. The ratio allowed for estimation and adjustment of power to the cells without measurement each time adjustment was required. These ratios were recorded and used in our LabView Cell Exposure software, which performs calculations and drives the x-y translational stages for exposures across the microtiter plates. The telescope is comprised of a combination of lenses that focus the beam to the hole in the annular mirror (silver coated on an aluminum substrate) while providing a 4 mm beam diameter at the level of the cells.

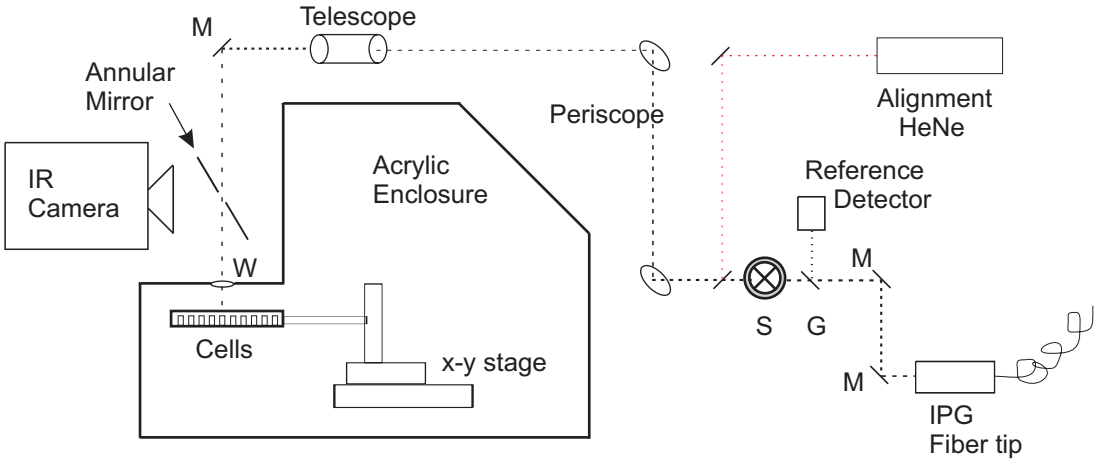

Figure 1. A schematic is provided which illustrates the apparatus for positioning, exposing, and obtaining thermal images of samples for laser exposure experiments. (M, mirror; S, mechanical shutter; G, BK7 glass; W, optical window)

The acrylic enclosure shown in Figure 1 was constructed from 0.5 inch Plexiglas and provided the work space for laser exposure of cultured cells. The enclosure provided both temperature (35 +\- 1 ^{o}C) and relative humidity (50% – 60%) control using a high-capacity, air-driven heater and a cartridge heater placed in a container of water, respectively. A ledge along the back of the acrylic box provided a means of imaging the corneal cells with an IR camera (through a MgF_2 window) during laser exposures. Laser beam diameter was measured, in two axes, using the knife-edge technique with 86% and 14% total power as clip points.[3]

Corneal simulants (air-lifted hTCEpi cells on growth inserts) were twice rinsed in pre-warmed (37 ^{o}C) Hank's Balanced Salt Solution (HBSS), equilibrated in the environmental chamber with 0.5 ml HBSS in the lower chamber (under the membranes), and exposed to the laser (0.25 s each). The HBSS beneath the inserts was then replaced with fresh KGM-2 medium (37 ^{o}C) and the simulants were allowed to "recover" for 1 hr at normal simulant culture conditions. A broad range of irradiance was chosen in the first round of exposures, and the results obtained from them allowed later for narrower ranges of irradiance exposures in an optimized process for determining ED_{50} thresholds.

2.3 Infrared Imaging

IR images were obtained using a FLIR model SC4000 camera with a 100mm InSb lens with f/2.3 and Bayonet Mount Optical Interface (322-0005-04, FLIR). Extender rings from a set (222-0006-05, FLIR) were used to increase the distance between the 100 mm lens and camera by 1.5". The field of view (FOV) and working distance for our imaging

setup were measured to be approximately 21 mm and 270 mm, respectively. Table 1 provides key settings for the thermal camera during experiments.

pixel array size	192 x 192
frame rate	800 Hz
integration time	0.360 ms
global offset	1800
global gain	1

Table 1. IR camera settings during calibration and experimental data collection

Thermal calibration was conducted using a portable blackbody source (M316, Mikron) with NIST traceable calibration from ambient + 10 °C to 110 °C. Thermal camera temperature calibrations were conducted with the blackbody surface at the image plane of the thermal camera, within the experimental setup. Two calibration curves were collected for a temperature range of 30-110C, with the acrylic enclosure at room temperature. A final curve was collected from 40-110C, with the acrylic enclosure temperature set to 35 C and 60% relative humidity (experimental conditions). Curve fit software (TableCurve 2D V5.01, SYSTAT) was used to determine the relationship between temperature and pixel value for both data sets. No significant differences were found between the data sets. For this reason, all data were lumped and used for determination of coefficients for various curve fits. The function [y =a+b*ln(x)/x] was chosen as the best fit with easiest implementation for use with SAF analysis, where x is pixel value and y is temperature value in degrees C. Values for a and b were experimentally determined to be 162.884 and -81808, respectively. R-squared value for this fit was 0.9990.

To assist in rapid data analysis, a software tool (SAF analysis) designed using LabView (National Instruments) was developed that can read movie files generated by RDAC, the FLIR software associated with the thermal camera. SAF analysis utilizes existing NUC files (RTools, FLIR) to correct image non-uniformity, bad pixels, and offset. The program calculates average, max, min, and standard deviation temperature values for a region of interest (ROI) or line section, based on curve-fit functions. The curve-fit functions can be selected and/or modified by the user based on calibration data. Maximum (peak) temperature values were extracted from cell culture data sets using the curve-fit function mentioned above and applied non-uniformity correction (NUC) obtained using FLIR software (RTools, FLIR) in SAF analysis. Extraction was limited to a circular ROI of diameter 5.2 mm, which was manually placed over the laser exposure site. The temperature output from SAF analysis assumes that sample emissivity is equal to the calibration blackbody. In this case, the blackbody emissivity was 0.996. We experimentally determined the emissivity of membranes submerged in HBSS to be 0.88 +/- 0.01. We assume the emissivity of corneal simulants to be equivalent to that of submerged membranes, due to their high water content and the lack of other intrinsic absorbers in the IR regime. This emissivity value is consistent with measured emissivity of cornea in the 3-5 µm band, which has been measured *in vivo* for porcine and rabbit cornea, 0.84 and 0.83 respectively.[4, 5] Therefore, all final temperature values from SAF Analysis are divided by 0.89 to correct for emissivity of the cells prior to data analysis.

2.4 Analyses for Thermal Damage to Membrane Inserts

Irradiance-dependent temperature increases from exposure to the 2-µm laser were measured using the IR camera during laser exposure to membranes that were dry, wetted from below with HBSS then suspended in air, and wetted from below with HBSS (Figure 5). Additionally, confocal microscopy (Mitutoyo 50x long-working distance microscope objective) was used to check for observable morphological changes in laser-heated and control membranes. Here, the center of one membrane (wetted with HBSS from below during exposures) was subjected to repeated exposure with the 2-µm laser (20 exposures in all) at irradiances of 5 - 25 W/cm^2 (increments of 5 W/cm^2) with four replicates each. Thermal profiles were recorded with the IR camera for each of the 20 exposures (data not shown).

2.5 Assay for Cytotoxic Effects due to Laser Heating of Membrane

Samples were tested for direct cytotoxic chemical release during membrane heating and for whether heating membranes to 100 °C would lead to reduced plating efficiency for the corneal cells. A small volume of hot (100 ºC) HBSS or water was added to the wetted membrane of inserts and, after various times, the soluble fraction was added (mixed 1:5 with KGM-2 medium or directly [HBSS]) to healthy corneal cells growing on plastic. Cell viability was determined after

overnight incubation with complete medium by staining with calcein-AM and ethidium homodimer1 and counting live and dead cells in three different fields of view under the 40x microscope objective. The heat-treated and control membranes were then seeded with healthy corneal cells, whose adherence was judged by staining for viability with trypan blue, then counting stained (dead) and total cell numbers under the 40x microscope objective. These results were analyzed to determine whether the heat-treated insert adversely influenced cell attachment with respect to control.

2.6 Assay for Laser Damage to Corneal Simulants

Following exposures, the inserts were replaced in a 24-well plate containing complete growth medium to incubate at 37 °C for 1 hr prior to stain. Cells were assayed for viability using 1.7 μM calcein-AM and 1.4 μM Ethidium homodimer 1 (EthD1) in 0.1 mL HBSS (10 min at 37 ºC), followed by gentle washing twice with HBSS. Damage was scored positive when the monolayer region central to the insert was devoid of staining by calcein-AM (bandpass exciter of 460-490 nm and a bandpass emitter of 490-530 nm). Fluorescence of nuclei with EthD1 (bandpass exciter of 475-545 nm and a barrier filter at 590 nm) provided confirmation of damage. Images were taken for documentation (individual red and green channels, phase-contrast, and bright-field).

Scoring of damage (yes/no) was performed (blind of dosimetry) by three individuals where the final determination required a consensus from two. These binary data were input into the Probit software package.[6,7] In addition to probability-dose information (ED_{50}), the Probit output includes uncertainty intervals (fiducial limits at 95% confidence) related to the ED value, and the Probit slope (first derivative at a probability of 0.5 for ED_{50}). In this study, the ED_{50} is defined as the dose irradiance that results in loss of viability (cell death) with a likelihood of 50% (i.e. corneal simulants will die 50% of the time when exposed to the ED_{50} irradiance).

3. RESULTS

3.1 Cell Culture

At the start of this study, cells were grown on membranes and inserts with various seeding densities and for various growth periods without airlifting (data not shown). These cultures grew well, but demonstrated a dramatic reduction in viability when culture was extended beyond ten days (Figure 3). On the contrary, cells that were airlifted retained good viability throughout the 14 day growth period. For this reason, we adopted conditions originally used by Robertson *et al.* (16,600 cells per insert, as described in Methods and reference 1) because of the complexity introduced into the tissue by extended growth period (14 days total) and air lifting (the last 7 days). A microscopic analysis of the cells on the control inserts from laser experiments is shown in Figure 2. The corneal simulants are complex, having an abundance of what appears to be pigmentation, multiple layers of cells, and swirls of tissue. These properties are found in approximately the same proportions in all three control insert samples. This complexity of the simulants made viability and cell density assessment problematic. Although it is difficult to see in the images, there seems to be a large number of dead cells (red), but the number of live cells (green) far outnumbers them, thus there is good percent viability. The improved stability and favorable properties of corneal cells grown on membranes with air-lifting, when compared to corneal cells grown on plastic without air-lifting, indicates a requirement for extended time of air-lifting to obtain the modification in cell behavior and properties required to obtain stable corneal simulants.

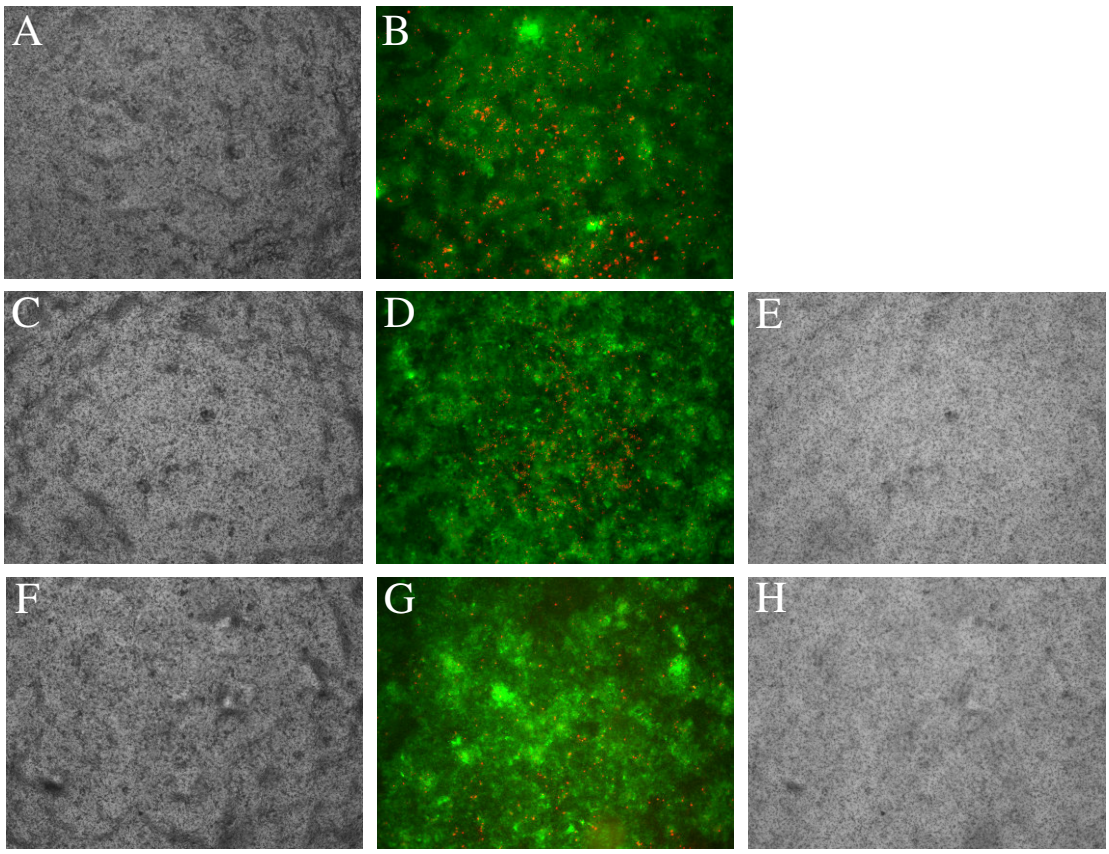

Figure 2. Microscope images of hTCEpi cell simulants. Three control wells (A – B, C – E, and F – H) for the laser experiments were imaged at 4x magnification as either phase contrast (A, C, F), overlay of calcein and ethidium (B, D, G), or bright field (E, H).

3.2 Assays for Chemical Alterations During Membrane Heating

To identify cytotoxic consequences of heating the membrane of a growth insert as the result of laser irradiation, boiling HBSS or water was applied to the membrane. The resulting inserts and buffer were then assayed for inhibition of cell attachment and reduced cell viability, respectively. Table 2 summarizes the results of these experiments. In most cases, there was no significant difference between controls and heat-treated conditions. However, viability did appear to be somewhat decreased for cells exposed to HBSS from heat-treatment (Table 2, experiment 3). We believe this decrease can be explained by the increased cell number present for those conditions. These cells, when grown on plastic, demonstrate reduced viability when total cell number increases. This effect can be seen in Figure 3 below.

		Total Cells	STD cells	% Viability	STD % Viability
Cells grown on heat-treated inserts	Control HBSS[1]	83	2.5	90	1.1
	Hot HBSS[1]	84	4.9	90	1.2
	Control Water[2]	81	5.5	87	1.0
	Hot Water[2]	90	5.1	85	1.5
Cells exposed to HBSS or water used to heat-treat inserts	Growth Medium[3]	85.2	2.1	94.3	0.8
	HBSS[3]	86.5	2.1	91.4	2.0
	HBSS used to heat-treated insert[3]	90.8	2.6	87.5	2.2
	Growth Medium[4]	84.3	3.3	94.7	1.3
	Water diluted 1:5 in HBSS[4]	83.7	1.8	94.4	1.2
	Water used to heat-treat insert diluted 1:5 in HBSS[4]	85.0	3.6	93.5	0.8

Table 2. Total cell number and viability with standard deviations presented for 4 different experiments (designated by superscripts after the labels for each experimental condition). The top portion contains data collected following 1-2 days growth on control and heated membranes, with experiment 1 exposed to room temperature or heated HBSS and experiment 2 exposed to room temperature or heated water. The bottom portion contains data collected following initial growth of cells on inserts with either GM only, unheated HBSS or water diluted 1:5 in HBSS, and heated HBSS (experiment 3) or heated water diluted 1:5 in HBSS from membrane heat-treatment (experiment 4).

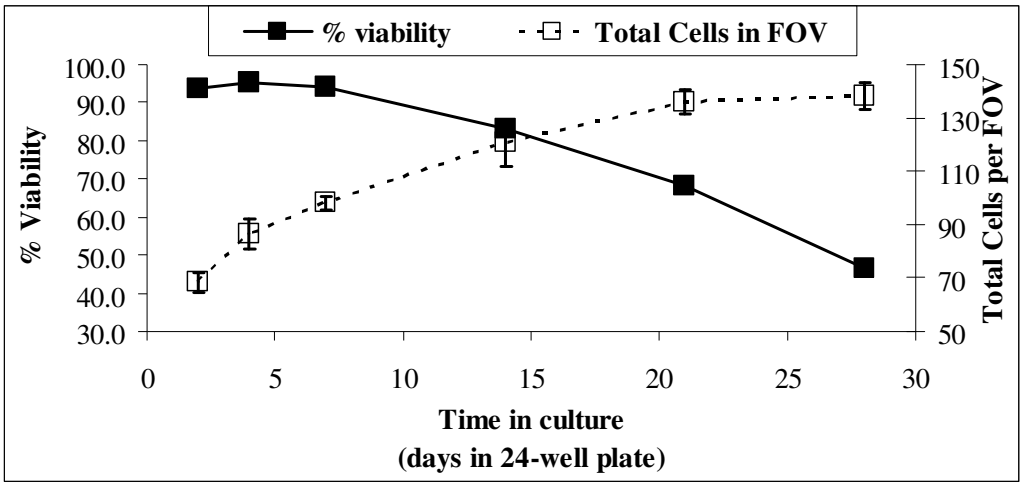

Figure 3. Viability and Total Cell number data for cells grown on 24 well microtiter plates.

Also, an insert membrane was repeatedly exposed to the 2-μm laser in the same site, and then compared to an unexposed insert via transmitted light microscopy using a 50x Mitutoyo long-working-distance microscope objective with 4x digital zoom (200x effective magnification). There was no discernable change in the microscopic appearance of the membrane due to repeated laser exposures (Figure 4).

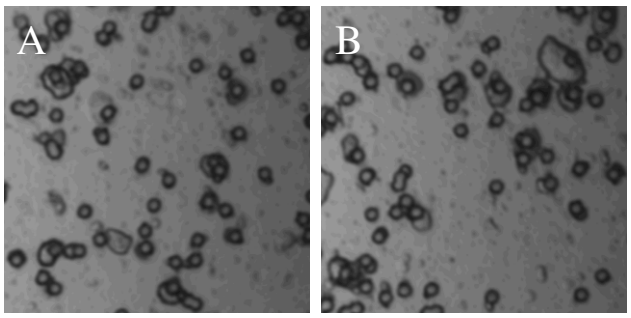

Figure 4. Confocal microscopy is provided for an unexposed insert membrane (A) and a laser-heated insert membrane (B). Images were taken with transmitted light using a 50x Mitutoyo objective at digital zoom of 4x (total magnification 200x).

3.3 Laser Exposures to Inserts without Cells

Three membrane conditions were examined to determine optimum conditions for obtaining thermal data from cell culture (Figure 5). On dry membranes, laser exposures resulted in minimal temperature rise. We interpret this lack of heating to low absorption by dry membranes at the laser wavelength. This is encouraging, as we wished to avoid heating effects generated by non-cell sources. Results from wetted membranes suspended in air are somewhat scattered and have a low R squared when fit with a first-order linear trendline. These results are indicative of inconsistent presence of media at the membrane surface after wetting. This experimental condition would be detrimental to the accurate measurement of temperature profiles in cell culture, simply due to the unreliable absorption characteristics of the cell substrate (membrane) during laser exposure. Wetted membranes left in full contact with HBSS demonstrated a linear thermal profile with peak temperatures approaching boiling at higher irradiances and good R^2 (0.983). This is believed to be due to high water absorption at the laser wavelength.

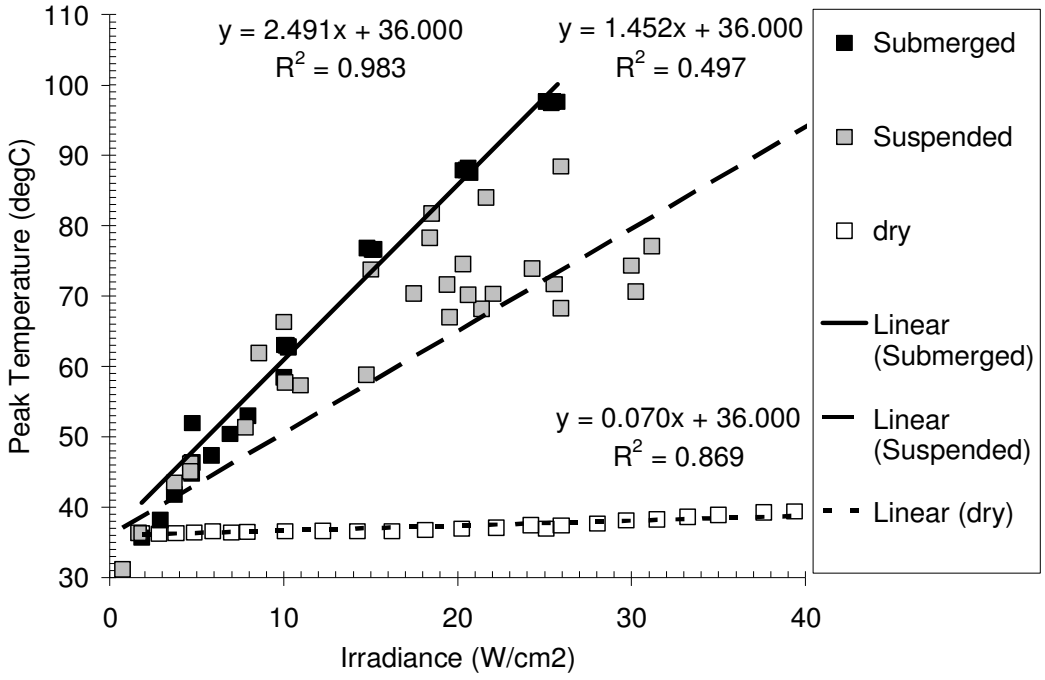

Figure 5. Peak temperature during laser exposure is obtained from thermal images and plotted against irradiance. Three membrane conditions were examined: wetted with HBSS with full contact from below (submerged), wetted then suspended in air (suspended), and dry (as received from manufacturer). Trendline y-intercept values were forced to 36°C (average temperature of the thermal box) for ease of comparison.

3.4 Laser Exposures to Corneal Simulants

Corneal simulants were exposed to 0.25 sec of 2-µm laser irradiation (4 mm beam diameter) at various irradiances. Cell viability was determined 1 hr after exposure to yield an ED_{50} threshold value (Figure 6). The threshold value of 12.7 W/cm^2 is comparable to the reported[4] *in vivo* rabbit corneal threshold (9.5 W/cm^2), as calculated based on threshold power (in milliwatts) and laser diameter provided. Real-time IR imaging provided thermal profile data and peak temperature was selected as the metric for analysis. The peak temperature of the simulants at the threshold ED_{50} irradiance was estimated to be 77.5 °C, with lower and upper bounds of 60.9 °C and 91.2 °C, respectively (Figure 7). Lower and upper bounds were defined as the peak temperatures that correlated with the upper and lower fiducial limits at the ED_{50} irradiance. This compares with existing *in vivo* rabbit corneal threshold data[5], where the threshold temperature was within the range of 64.6 °C to 68.9 °C. It should be noted that the *in vivo* threshold upper and lower bounds are defined as the maximum peak temperature with consistent absence of damage and the minimum peak temperature with consistent presence of damage, respectively. Finally, irradiance dependence of heating due to 2-µm laser exposure was compared for inserts that were wetted with HBSS from below and corneal simulants grown on inserts and wetted with HBSS from below (Figure 8). The trends indicate that no significant difference exists between the thermal properties of wetted inserts when compared to corneal simulants grown on wetted inserts, with respect to exposure to 2-µm laser.

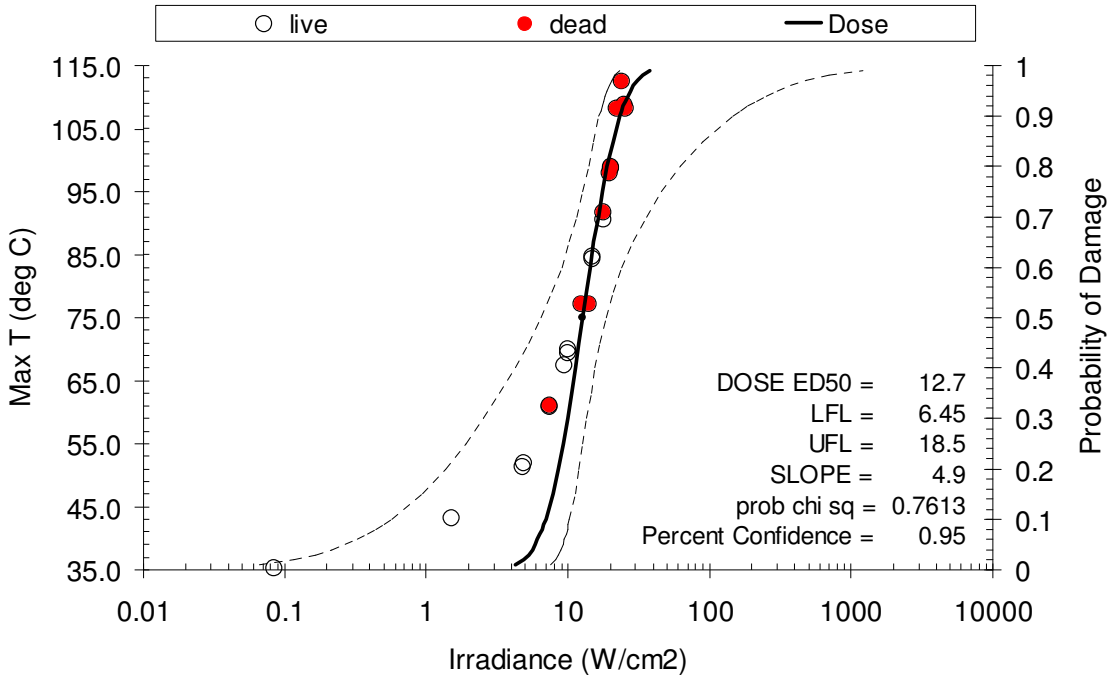

Figure 6. Data shown for cell viability and maximum temperature rise versus laser irradiance. Unfilled dots indicate cells that remain viable after laser exposure. Filled dots indicate cells that were scored not viable by 2 out of 3 readers. The dark line delineates the dose value for various probabilities of damage as defined by Probit. This line is bound by two light dashed lines, which indicate the upper and lower fiducial limits (LFL and UFL).

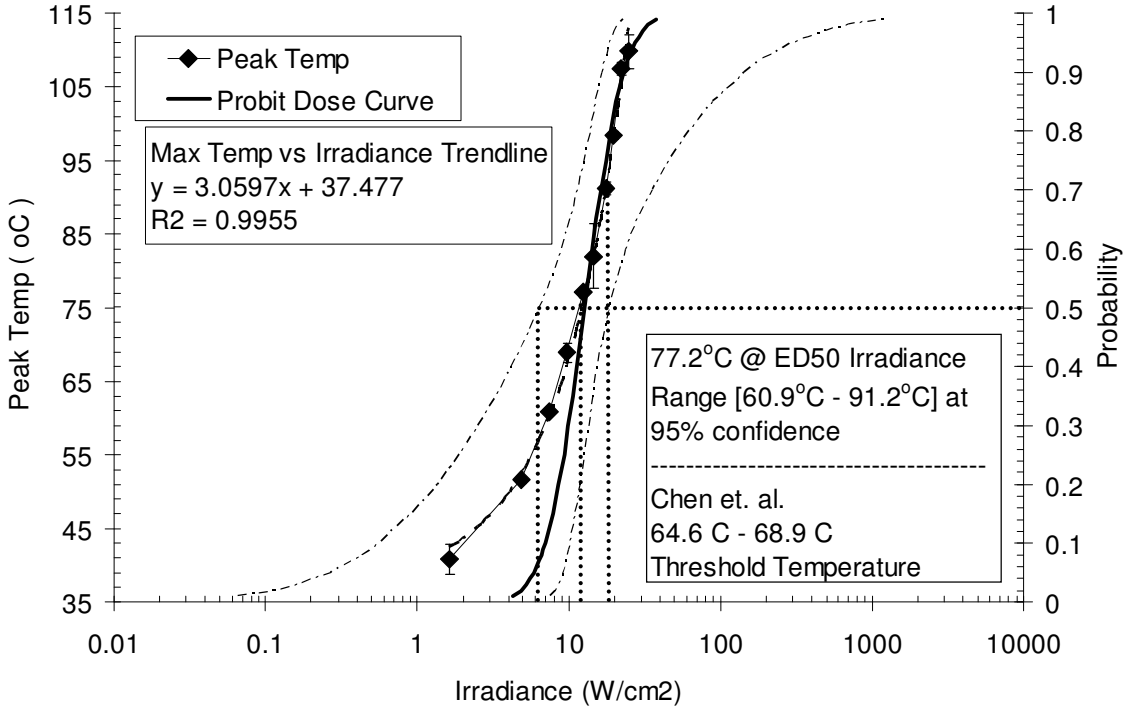

Figure 7. Data shown for cell viability and maximum temperature rise versus laser irradiance. The dark line delineates the dose value for various probabilities of damage as defined by Probit. This line is bound by two light dashed lines, which indicate the upper and lower fiducial limits (LFL and UFL). A horizontal dashed line is drawn though the estimated dose for 50% damage (ED_{50}) and vertical lines are extended from the horizontal, with the temperatures at dose, UFL, and LDL indicated for ED_{50} by the point where those lines pass through the temperature versus irradiance trendline.

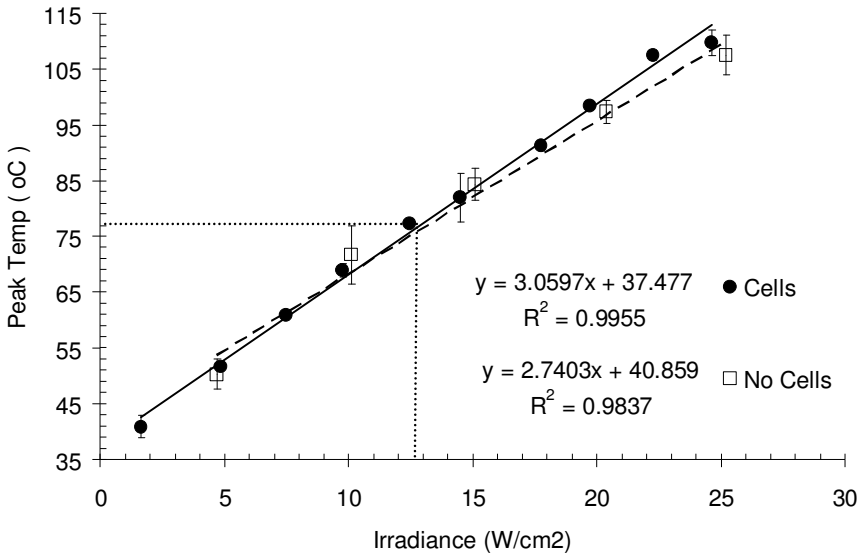

Figure 8. Peak temperature data from exposures used in the threshold ED_{50} determination. The peak temperature at the ED_{50} value of 12.7 W/cm^2 was 77.5 °C.

For comparison with existing *in vivo* rabbit corneal threshold data[5], peak temperature data was compared to laser power rather than laser irradiance (data not shown). The slope of the line generated from the corneal simulant data was 0.0243 °C/mW, which compares with the rabbit corneal value[5] of 0.029 °C/mW. A representative graph of the threshold peak temperature data set, the 12.5 W/cm^2 thermal profile, is provided (Figure 9). This graph presents peak temperature as a function of time. Thermal camera acquisition was started approximately 0.75 sec prior to laser exposure with 4 seconds duration. It should be noted that temperature rise occurs only during laser exposure (0.25 sec duration), followed by an exponential decay for the remainder of acquisition.

Figure 10 provides five examples of damage to corneal simulants exposed to the 2-µm laser irradiation. It should be noted that damage is readily identifiable in the phase contrast pictures, as a darkened region (Figure 10.D, F, H). Stain with ethidium / calcein was made somewhat interesting due to the multiple cell layers present, resulting in apparent co-staining (Figure 10.C, E).

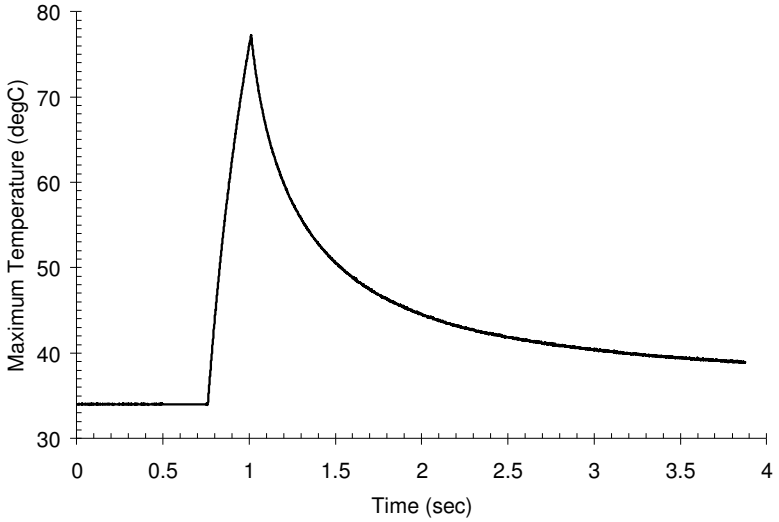

Figure 9. Thermal profile for the 12.5 W/cm^2 exposure in the threshold ED_{50} data set. The peak temperature was 68.7 °C, the same as the deduced threshold peak temperature rise for the entire data set.

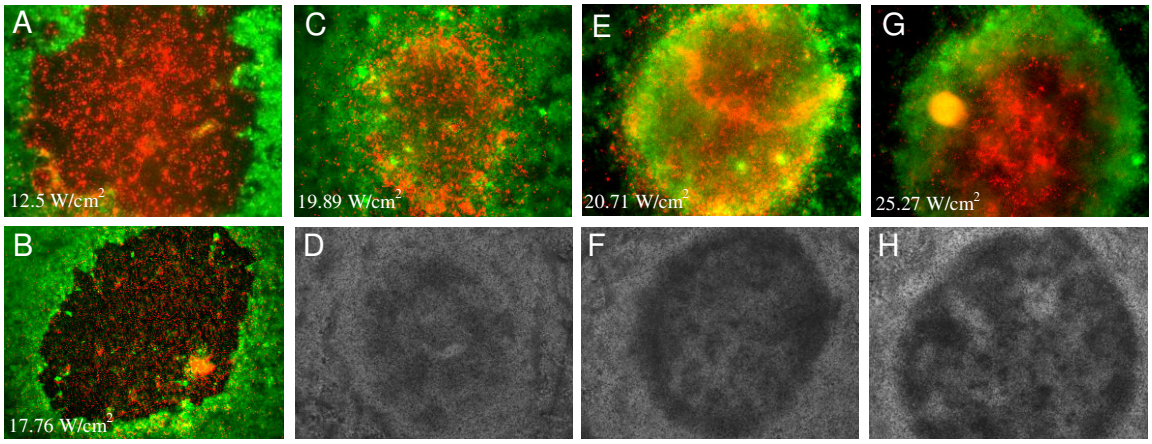

Figure 10. Corneal simulants were exposed to 2-µm laser irradiation (4 mm beam diameter) for 0.25 seconds. After incubation in normal growth conditions for 1 hour, simulants were stained with viability dyes and imaged using a 4x objective. Irradiances (W/cm^2) leading to the damage shown were 12.5 (A), 17.76 (B), 19.89 (C, D), 20.71 (E, F), 25.27 (G, H). The color overlays represent combined calcein-AM and ethidium homodimer staining (A, B, C, E, and G). Panels D, F, and H are phase contrast images from inserts represented by C, E, and G, respectively.

4. DISCUSSION

The use of corneal simulants, as generated in our labs, had advantages and disadvantages. The complexity of the tissue generated was thought to mimic the properties of the cornea. Since our methods were adopted from Robertson *et al*[2], we assume that many or all of the properties identified by Robertson *et al.* were present in our cultures. In brief, these properties include a well stratified epithelium (5 – 7 cell layers) with tight junctions in the apical layer(s). In the Robertson article[2], the corneal simulants were reported to be composed of differentiated cell types, which was then verified using laser scanning confocal microscopy with antibodies to key corneal stratification marker proteins, such as K3. Although no images were shown in the Robertson article[2], the authors showed a dramatic increase in the K3 protein levels in their cultures after 7 days of air lifting. The increased pigmentation evident in phase-contrast and bright-field images of our airlifted corneal simulants is likely due to the production of K3 keratin, while the dimpling appearance suggests multiple cell layers (Figure 2). For future experiments in our lab, we would like to perform detailed analyses of corneal simulants, to include determination of the thickness, number of layers present, and type of pigment evident in phase-contrast and bright-field images of the corneal simulants. Unfortunately, these analyses were not within the scope of this investigation. It should be noted that we were able to maintain the cells with good viability using the same methods described by Robertson *et al*. In addition, thermal images were obtained for each exposure, which could then be compared against phase-contrast, bright-field, and fluorescence microscopy images obtained after the fact (Figure 10).

Overall, we describe two lines of investigation. First, we took measures to ensure that our cultured corneal epithelial cells were injured due to a thermal process, rather than a chemical reaction as a result of heating of the porous membrane on which they were grown. Second, we determined an irradiance threshold for cell death using the 2-µm laser while relating this damage to a thermal response of the corneal simulants.

No significant inhibitory effects were observed when cells were plated onto insert membranes that had been heated with hot water or buffer, relative to controls (Table 3). Also, possible inhibitory effects were observed for cells exposed to HBSS or water diluted 1:5 in HBSS that had been used to heat treat membranes (Table 3), but they were negated by the inverse relationship between viability and total cell number when cells are grown directly on plastic well plates (Figure 3). Combined with the lack of ultrastructural damage to membranes (Figure 4) after repeated laser irradiation, our results indicate that cellular damage identified in our ED_{50} study is a result of direct heating of the corneal simulants.

Thermal profiles of the membranes inserts without and with corneal epithelial cells due to laser exposure were compared and no significant difference was found (Figure 8). This indicates that the very high absorption of water at 2 µm is likely the principal source of heating for both conditions. We chose to leave water beneath the membrane inserts for a number of reasons. First, in the eye, the cornea is bounded by the aqueous humor (directly behind the cornea) and an air interface. Leaving media beneath membranes provides a model system that more closely mimics *in vivo* conditions, because the corneal simulants would have both an air interface (top surface) and a liquid interface consisting of HBSS (bottom surface). Second, removal of media from beneath the membranes resulted in variable temperature rises (Figure 5, suspended), which we attribute to variable water content behind the cells, resulting in inconsistent absorption properties at the laser wavelength. Finally, the presence of media beneath the membrane ensures that the cells remain moist and viable for the duration of the experiment.

The *in vitro* ED_{50} value of 12.5 W/cm^2 is similar to the *in vivo* value of 9.5 W/cm^2 described by Chen *et al*[5]. Fiducial levels were not as narrow as we typically achieve with *in vitro* studies due to low sample numbers (3 plates over 2 days). In the future, we hope to revisit this study and collect more data for input to Probit. That said, the ED_{50} value (9.5 W/cm^2) of Chen *et al*[5]. falls within the *in vitro* ED_{50} lower and upper fiducial limits at 95% confidence (6.45 W/cm^2 and 18.5 W/cm^{2,} respectively). There are, however, some differences in methodology between these two experiments that should be recognized. First, the cultured simulants from our study are derived from human corneal epithelial cells and the animal study of Chen *et al*[5] used rabbits. The animal study used visible opacity as an endpoint while we used fluorescent indicator dyes for cell viability. The phase-contrast images from Figure 10 are suggestive that damage inflicted by the laser would cause sufficient change in light transmission to cause opacity if appropriately assayed. Additionally, our setup provided a reasonably flat surface for laser interaction whereas the rabbit eye has a defined curvature. It should be noted that both groups used the same laser wavelength, exposure duration, and spot diameter for exposures. Also, the expected water-dependence of the 2-µm laser-tissue interaction (membrane alone versus membrane with cells) is demonstrated, suggesting that little difference is likely to exist between the i*n vivo* and *in vitro* studies.

Comparing two additional data points, peak temperature (Tp) threshold and the slope of Tp versus laser power, also indicates that our *in vitro* system approximates corneal organ tissue for 2-µm laser damage evaluation. The *in vitro* Tp value at the ED_{50} (77.5 °C) with a range designated by the upper and lower fiducial limits (60.9 °C and 91.2 °C) encompasses, but is slightly higher than, the range identified by Chen *et al.* (64.6 °C – 68.9 °C), while the slopes for the linear fit lines (0.0243 °C/mW *in vitro* and 0.029 °C/mW *in vivo*) are within reasonable agreement. A major note is that the measure of Tp threshold upper and lower bounds were defined in the animal study as the lowest temperature that consistently lead to damaged cornea and the highest temperature that consistently did not lead to damaged cornea, while our assessment of Tp threshold was the temperature at the upper and lower fiducial limits about the ED_{50} irradiance as obtained using Probit. While these are both valid methods for providing upper and lower temperatures that result in threshold, our definition of the threshold as the point at which damage occurs 50% of the time could result in consistently higher upper and lower limits when compared to the method used in the animal study. Also, additional data collection in future studies could provide tighter fiducial limits around the ED_{50}, providing a narrower range of temperatures that results in damage 50% of the time.

We conclude that our *in vitro* corneal simulants approximate corneal tissue of live animals to the extent that they could be used in future experiments involving laser-tissue interactions.

5. REFERENCES

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